DESATURASE DELTA-6 mRNA EXPRESSIONS IN HIGHLY UNSATURATED FATTY ACIDS BIOSYNTHESIS PATHWAY DURING FOLLICLE MATURATION IN FEMALE ZEBRAFISH (*Danio rerio*)

by

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-Sairatul Dahlianis Ishak-

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LIST OF ABBREVIATIONS

Acetyl CoA	acetyl Coenzyme A
ANOVA	analysis of variance
ARA	arachidonic acid
ATP	adenosine triphosphate
BLAST	basic local alignment search tool
cDNA	complementary deoxyribonucleic acid
C_T	threshold cycle
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
DT	DNase treatment
EPA	eicosapentaenoic acid
FAME	fatty acid methyl esters
fadsd6	delta-6 fatty acyl desaturase
GC	gas chromatography
h2afz	H2A histone family
HUFA	highly unsaturated fatty acids
IPTG	isopropyl β-D-thiogalactopyranoside
LA	linoleic acid
LB	Luria-Bertani
LNA	linolenic acid
MMLV-RT	Moloney murine leukemia virus-reverse transcriptase
MOPS	4-morpholinepropanesulfonic acid
mRNA	messenger ribonucleic acid
PUFA	polyunsaturated fatty acids
RNA	ribonucleic acid
RT-PCR	reverse trancriptase polymerase chain reaction
rRNA	ribosomal ribonucleic acid
SEM	standard error of mean
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

PENGEKSPRESAN mRNA DELTA-6 DESATURASE DALAM KITARAN BIOSINTESIS ASID LEMAK SANGAT TIDAK TEPU SEMASA KEMATANGAN FOLIKEL IKAN BETINA ZEBRAFISH (*Danio rerio*)

ABSTRAK

Sangat sedikit yang diketahui tentang sintesis asid lemak tidak tepu seperti asid eikosapentanoik (EPA, C20:5*n*-3), asid dokosaheksaenoik acid (DHA, C22:6*n*-3) dan asid arakidonik (ARA, C20:4*n*-6), atau secara berkumpulan lebih dikenali sebagai asid lemak sangat tidak tepu (HUFA), di dalam ovari ikan walaupun ia memainkan peranan yang sangat penting dalam pembiakan vertebrata ini. Ikan zebrafish (*Danio rerio*) mempamerkan keupayaan untuk mensintesis ketiga-tiga HUFA ini melalui kitaran yang melibatkan desaturasi dan elongasi dua prekursor iaitu asid linoleik (LA, C18:2*n*-6) dan asid linolenik (LNA, C18:3*n*-3). Bagi lebih memahami kepentingan serta pengawalaturan sintesis HUFA dalam ovari, kajian ini menunjukkan corak ekspresi mRNA untuk desaturase (*fadsd6*), iaitu satu enzim perlu yang terlibat dalam kitaran biosintesis HUFA dalam lima peringkat folikel ovari zebrafish yang berbeza iaitu pre-vitelogenik, vitelogenik awal, vitelogenik akhir, kematangan dan ovulasi. Aras mRNA bagi *fadsd6* dalam peringkat-peringkat folikel ovari yang berbeza ini ditentukan oleh kaedah esei RT-PCR. Di samping itu, profil asid lemak tidak tepu bagi setiap peringkat folikel juga dianalisa menggunakan gas kromatografi. Keputusan esei-esei RT-PCR menunjukkan bahawa desaturase telah mempamerkan peningkatan kawalaturan yang bermakna semasa peringkat kematangan oosit berbanding peringkat-peringkat yang lain. Pada masa yang sama, analisis komposisi asid lemak tidak tepu menunjukkan bahawa aras ARA secara bermakna paling tinggi pada peringkat folikel pre-vitelogenik dan kematangan. Aras DHA pula paling tinggi di peringkat vitelogenik akhir dan kematangan. Secara

keseluruhannya, keputusan ini mencadangkan kewujudan satu sistem sintesis HUFA yang bertanggungjawab untuk mensintesis HUFA bagi menggalakkan kematangan oosit dan mungkin juga proses-proses ovulasi. Kelebihan menggunakan ikan zebrafish sebagai sistem model untuk memahami asal-usul folikel adalah berguna bagi lebih menghuraikan aspek-aspek kawalaturan dan mekanisme sintesis HUFA dalam ovari.

DESATURASE DELTA-6 mRNA EXPRESSIONS IN HIGHLY UNSATURATED FATTY ACIDS BIOSYNTHESIS PATHWAY DURING FOLLICLE MATURATION IN FEMALE ZEBRAFISH (*Danio rerio*)

ABSTRACT

Very little is known about the synthesis of unsaturated fatty acids such as eicosapentaenoic acid (EPA, C20:5*n*-3), docosahexaenoic acid (DHA, C22:6*n*-3) and arachidonic acid (ARA, C20:4*n*-6), or collectively known as the highly unsaturated fatty acids (HUFA), in the fish ovary although they play pivotal roles in this vertebrate reproduction. The zebrafish (*Danio rerio*) display capability to synthesize all three HUFA via pathways involving desaturation and elongation of two precursors, the linoleic acid (LA, C18:2*n*-6) and linolenic acid (LNA, C18:3*n*-3). In order to gain understanding on the importance and regulation of ovarian HUFA synthesis, this study shows the mRNA expression pattern of desaturase (*fadsd6*), an essential enzyme involved in HUFA biosynthesis pathway, in five different zebrafish ovarian follicle stages; pre-vitellogenic, early vitellogenic, late vitellogenic, maturation and ovulation stages. The mRNA levels of *fadsd6* in different ovarian follicle stages were determined by semi-quantitative RT-PCR assays. Concurrently, the fatty acid profile of each follicle stage was also analysed using gas chromatography. Results of RT-PCR assays have shown that desaturase displayed significant upregulation in expression during the oocyte maturation stage compared to the other follicle stages. At the same time, fatty acid composition analysis of different ovarian follicle stages also showed that ARA level was significantly highest in pre-vitellogenic and matured follicles. DHA level was highest in both late vitellogenic and maturation stage. Collectively, the findings seem to suggest the existence of a HUFA synthesis system, which could be responsible for the synthesis

of HUFA to promote oocyte maturation and possibly ovulation processes. The many advantages of zebrafish as model system to understand folliculogenesis will be useful platform to further elucidate the regulatory and mechanism aspects of ovarian HUFA synthesis.

CHAPTER 1

INTRODUCTION

1.1 Research background

Broodstock nutrition or dietary nutrient requirement during finfish reproduction is a main concern in aquaculture. It directly affects various reproductive parameters such as time to first maturity, gonadal development, fecundity, quality of eggs based on chemical composition, hatchability; and larval survivorship (De Silva and Anderson, 1995; Izquierdo *et al.*, 2001; Watanabe and Vassallo-Agius, 2003). Numerous studies were done in the interest of fish farmers to fulfill the overall broodstock nutrient requirement for greater economic return. The major dietary nutrients that provide energy to sustain fish metabolism, growth and reproduction are carbohydrates, proteins and lipids; with specific nutrients like essential fatty acids, acid aminos, carotenoids, acid ascorbics and vitamin E having key roles in regulating various reproductive processes (De Silva and Anderson, 1995; Izquierdo *et al.*, 2001; Watanabe and Vassallo-Agius, 2003).

Polyunsaturated fatty acids (PUFA) have long been known to play a vital role in fish reproduction. Both the *n*-6 and *n*-3 PUFA influence reproductive processes through a variety of mechanisms, which includes provision of precursors for prostaglandin synthesis, inducement of steroidogenesis and regulation of transcription factors involved in reproductive process (Wathes *et al.*, 2007). Such PUFA like the eicosapentaenoic acid (EPA, C20:5*n*-3), docosahexaenoic acid (DHA, C22:6*n*-3) and arachidonic acid (ARA, C20:4*n*-6); also collectively known as highly unsaturated fatty acids (HUFA) have been extensively studied for their importance in regulation of oocyte maturation and ovulation (Tahara and Yano, 2004; Pickova *et*

al., 2007). In aquaculture, supplement of HUFA in broodstock diet is essential to increase probability of spawning success (Izquierdo *et al.*, 2001; Watanabe and Vassallo-Agius, 2003). *In vitro* studies using teleost follicles have reported the stimulation of maturation and ovulation by ARA (McEvoy *et al.*, 2000; Patino *et al.*, 2003). Besides the provision of adequate dietary ARA, EPA and DHA levels respectively, studies have also demonstrated the importance of having a balanced dietary ratio of these three HUFA for better reproductive performance (Izquierdo *et al.*, 2001; Watanabe and Vassallo-Agius, 2003). Studies have shown that several marine fish species selectively transfer HUFA from muscle reserves to oocytes as preparation of long spawning season (Garrido *et al.*, 2007; Huynh *et al.*, 2007). In addition, comparative analysis of fatty acid composition in ovary of wild and captive farmed fish have proposed inferior or imbalanced ratio of HUFA as the main reason for poor spawning performance of farmed broodstock (Cejas *et al.*, 2003; Pickova *et al.*, 2007).

Freshwater fish species have the capacity to synthesize EPA and DHA from linolenic acid (LNA, 18:3*n*-3) and ARA from linoleic acid (LA, 18:2*n*-6) respectively, through two separate pathways involving desaturation and elongation of their respective precursors; unlike their marine counterparts which are incapable of synthesizing these HUFA *de novo* (Tocher *et al.*, 2002; Zheng *et al.*, 2004a). The extent to which fish can convert LNA and LA, to HUFA differs according to species and depend on the activities of the desaturase and elongase enzymes. In most freshwater fish, EPA is synthesized from LNA by desaturation at the Δ6 position, followed by a 2-carbon elongation, and a further desaturation at the Δ5 position. Subsequently, synthesis of DHA from EPA is believed to proceed via a C24 intermediate, requiring two successive elongations to 22:5*n*-3 and 24:5*n*-3, followed

by desaturation at the Δ6 position, and lastly a chain shortening process to produce DHA (Tocher, 2003). Production of ARA involves desaturation at Δ6 position of LA followed by a 2-carbon elongation process and lastly a desaturation at the Δ5 position (Tocher, 2003). Previous studies have reported the presence of desaturase and elongase mRNA in oocytes of two freshwater fish species, swordtail and zebrafish (Ling *et al.*, 2006; Jaya-Ram *et al.*, 2008). However, very little is known about the existence, role and regulation of the HUFA synthesis system throughout the follicle maturation process in the ovary (Wiegand, 1996). Characterizing and understanding this HUFA synthesis system, more importantly the working of desaturation and elongation will help to meet the requirements of HUFA in broodstock nutrition.

Zebrafish (*Danio rerio*) has gained popularity as model organism in developmental biology laboratories for studies like comparative phylogenetics and gerontology (Metscher and Ahlberg, 1999; Gerhard and Cheng, 2002). The many advantages of zebrafish as model system have also made this species favorable as a useful model to understand oogenesis and folliculogenesis (Ge, 2005). The female zebrafish has high fecundity and can generate large populations quickly; with the oocytes are easily differentiated to 4-5 distinct follicle stages throughout the follicle maturation process (Selman *et al.*, 1993; Gerhard and Cheng, 2002). In addition, *in vitro* maturation by inducing zebrafish oocytes with gonadotropin and steroid has also been developed (Selman *et al.*, 1994; Pang *et al.*, 2002a). This further facilitates the monitoring of oogenesis and folliculogenesis better, instead of relying on *in vivo* studies where one cannot see the maturation process in ovary. Accordingly, transcriptome and proteome analysis have been carried out to map vital molecular pathways responsible for both maturation and ovulation processes in zebrafish

(Knoll-Gellida *et al.*, 2006; Ziv *et al.*, 2008).

Based on the importance of HUFA in reproduction, the localized desaturation and elongation activities in oocyte could potentially be a source of HUFA for maturation and ovulation processes. This study will attempt to understand the importance and regulation of ovarian HUFA synthesis by characterizing the HUFA composition and mRNA expression of the desaturase enzyme involved in the HUFA biosynthesis pathway in different stages of the zebrafish ovarian follicle especially as they enter the maturation stage.

1.2 Objectives

To gain understanding of HUFA synthesis in the ovary, this study aims:

- To profile the fatty acid composition specifically the HUFA composition in the five ovarian follicle stages.
- To characterize the HUFA synthesis in ovary by tracking the expression of mRNA desaturase enzyme throughout the follicle maturation process.

CHAPTER 2

LITERATURE REVIEW

2.1 Lipid

As an essential nutrition in fish diets, lipids main function is either as high-energy storage molecules or as components of cell membranes. It provides energy for growth, reproduction, migration, and membrane structural components, whilst essential fatty acids and precursors of eicosanoids are required for regulatory processes and assist in the uptake of lipid soluble nutrients (McKenzie, 2001).

Lipids as defined by Christie (1982) are fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds, such as carotenoids, terpenes, steroids and bile acids. There are five major classes of lipids: fatty acids, triglycerides, phospholipids, sterols and sphingolipids (De Silva and Anderson, 1995). Fish lipids can be divided into two big groups; polar lipids that composed mainly of phospholipids and neutral lipids that composed principally of triglycerides (Tocher, 2003). Neutral lipids are formed by esterification of fatty acids with alcohol or glycerol; and although mono- and diacylglycerols can be found, triglycerides make up the greater part of neutral lipids in nature. Triglycerides or triacylglycerols, are aptly named since they are triesters of glycerols. Phospholipids are esters of glycerol, two of the alcohol groups are esterified with fatty acids and the third with phosphoric acid, which in turn is esterified by a nitrogenous base, the amino acid serine, the sugar alcohol inositol or by glycerol sulphate. The nature of the nitrogenous base provides the basis of the name for the specific families of phospholipids – phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and phosphoglycerides (Jobling, 1994).

2.1.1 Lipid metabolism

Metabolism is a whole range of biochemical processes that occur within any living organism which consists both of anabolism and catabolism (the buildup and breakdown of substances, respectively). The term is commonly used to refer specifically to the breakdown of food and its transformation into energy. Dietary intake of fish contains the major nutrients; proteins, carbohydrates and lipids. Generally, this process involves digestion, absorption, absorption and transport of nutrients. Feed consumed by fish are digested in the gut, absorbed by the gut lining and appear in the bloodstream as their component molecules. Proteins are digested to release their component amino acids, which are subsequently used to synthesize new proteins or for energy. Similarly, carbohydrates will be broken down to simple sugars (De Silva and Anderson, 1995).

Lipid is broken down to fatty acids. Following absorption, they are then resynthesized into lipids which form droplets. These lipid droplets are circulated in the fish blood system. In order to be used, they must again be broken down to their constituent fatty acids. Fatty acids are then used for synthesis of membranes or further degraded for energy. Lipids contain more energy per unit weight than any other dietary component and are used efficiently by fish as energy sources. Besides providing energy, they are source of hydrophobic components for the synthesis of macromolecules (Jump *et al.*, 1999).

The degradative pathways of acid aminos, simple sugars and fatty acids will eventually reach a common intermediate compound that is the acetyl coenzyme A (acetyl CoA). Acetyl CoA enters the citric acid cycle, which in turn is linked to the process of oxidative phosphorylation. The result is the production of CO_2, the

consumption of O_2 and the liberation of energy, which is then stored as high-energy phosphate molecules, adenosine triphosphate (ATP) (De Silva and Anderson, 1995).

2.2 Fatty acids

Fatty acids are defined as compounds synthesized in nature via condensation of malonyl coenzyme A units by a fatty acid synthase complex. They usually contain even numbers of carbon atoms in straight chains (commonly C_{14} to C_{24}), and may be saturated or unsaturated, and can contain a variety of substituent groups. It is a group of naturally occurring compounds, which have in common a ready solubility in such organic solvents as hydrocarbons, chloroform, benzene, ethers and alcohols.

Since fatty acids are carboxylic acids with long-chain hydrocarbon side group, the nomenclature of fatty acids follows a particular convention which is:

CX:Y*n*-Z

X denotes the number of carbon atoms, Y denotes the number of double bonds in the chain and Z denotes the carbon at which the first double bond appears numbering from the non-carboxyl end which is the methyl end ($-CH_3$). For example, C18:3*n*-3 indicates that this fatty acid posses 18 carbon atoms and have 3 double bonds at the position of carbon 3, calculated from the methyl end of the molecule (IUPAC-IUB, 1967; De Silva and Anderson, 1995). In popular jargon, *n* is often replaced by ω (the small letter greek omega) hence the term omega-3 and omega-6 fatty acids.

Fatty acids itself can be categorized into few groups which are the saturated fatty acids, moncunsaturated fatty acids or monoenes, polyunsaturated fatty acids (PUFA) and highly unsaturated fatty acids (HUFA). Saturated fatty acids are fatty acids without any double bonds like C16:0 and C18:0. Monoenes are fatty acids that

contain only a single double bond such as C18:1*n*-7 and C18:1*n*-9. The term PUFA refers to fatty acids that may contain two or more double bonds, whereas the term HUFA are fatty acids with carbon chain length more or equal to 20 carbon atoms and containing three or more double bonds (Christie, 1982).

2.2.1 Unsaturated fatty acid biosynthesis pathway

Linoleic acid (LA, C18:2*n*-6) and linolenic acid (LNA, C18:3*n*-3) are essential as the important HUFA in fish lipid nutrition such as arachidonic acid (ARA, C20:4*n-6*), eicosapentaenoic acid (EPA, C20:5*n-3*) and docosahexaenoic acid (DHA, C22:6*n-3*) are derived from them. However, vertebrates including fish cannot synthesize LA and LNA *de novo* as they lack the Δ12 and Δ15 fatty acid desaturase enzymes that are required for their production from oleic acid (C18:1*n*-9).

Nevertheless, it has been established that many vertebrate species can convert dietary LA and LNA to long chain *n*-6 and *n*-3 HUFA respectively (Sprecher *et al.*, 1995; Zheng *et al.*, 2004b). This includes freshwater fish species where LA and LNA are essential fatty acids in its diet, which means that these fatty acids are vital as they will affect their growth and reproductive parameters. Freshwater species has the ability to desaturate and elongate the 18 carbon atom fatty acids into HUFA according to their needs (Seiliez *et al.*, 2003; Turchini *et al.*, 2006). Unlike their counterpart, marine fish species lack this ability to convert LA and LNA to HUFA, thus EPA and DHA are regarded as essential fatty acids to marine species (Bell and Sargent, 2003).

The conversion of LA to ARA and LNA to EPA and DHA are done *in vivo* via two pathways by an alternating sequence of desaturation and elongation. These two pathways involved are as depicted in figure 2.1 (Agaba *et al.*, 2005). The LNA

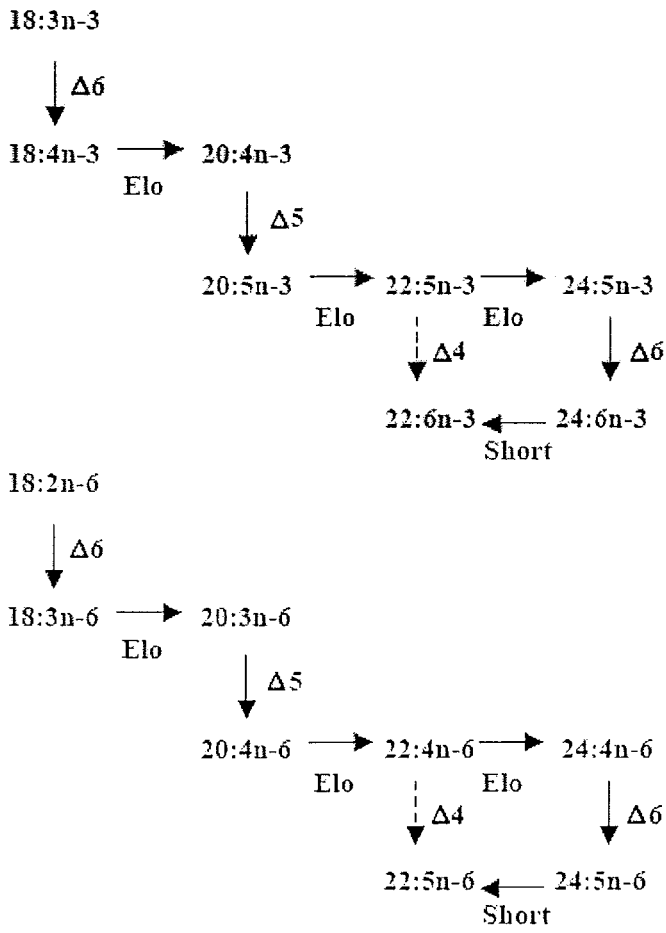

Figure 2.1: Pathways of highly unsaturated fatty acids (HUFA) biosynthesis from the C18 polyunsaturated fatty acids (PUFA), 18:3n-3 and 18:2n-6. Solid lines represent steps that have been shown to occur in fish Δ6, Δ5 and Δ4 fatty acid desaturases; Elo, fatty acid elongases; Short, peroximal chain shortening (adapted from Agaba *et al.*, 2005).

which is C18:3*n*-3; is desaturated to C18:4*n*-3 by Δ6 desaturase enzyme. Then, C18:4*n*-3 is elongated to C20:4*n*-3 and subsequently subjected to desaturation by Δ5 desaturase to become C20:5*n*-3 or EPA. Hypothetically, the insertion of the last Δ4 double bond in the end product which is DHA or C22:6*n*-3 is thought to occur through Δ4 desaturase enzyme from its immediate precursor C22:5*n*-3. However, a study in rat liver microsomes has shown that the EPA is elongated twice to C22:5*n*-3 and then C24:5*n*-3, before it is desaturated to C24:6*n*-3 by Δ6 desaturase. This C24:6*n*-3 precursor undergoes peroximal chain shortening to become DHA (Sprecher *et al.*, 1995). As for the *n*-6 pathway, a similar sequence of desaturation and elongation happens where the end product is C22:5*n*-6 instead of DHA in the *n*-3 pathway. In these two pathways, researchers have given particular emphasis in the production of the three HUFA, which are EPA, DHA and ARA, as they act as precursors for eicosanoids, a physiologically dynamic group of molecules responsible for an array of cellular activities including gene regulation, signaling and maintenance of membrane integrity. Prostaglandins, the oxygenated metabolites of ARA and EPA have been shown to play essential roles in the development of vertebrates (Cha *et al.*, 2006).

2.2.2 Desaturase and elongase genes in the HUFA biosynthesis pathway

Studies in genetics and molecular level have been carried out to explain the fatty acid metabolism in fish through cloning, functional characterization and gene expression of desaturase and elongase genes. Desaturase and elongase gene expressions are affected by dietary fatty acid treatments in fish in which several studies has shown that by manipulating the levels of dietary fatty acids will cause

increasing activities of the fatty acyl desaturation and elongation pathway in isolated hepatocytes (Tocher *et al.*, 1997; Zheng *et al.*, 2004a, 2004b).

A zebrafish EST desaturase sequence has been identified and was found to have 64% and 58% similarity with human Δ5 and Δ6 desaturase genes respectively (Hastings *et al.*, 2001). This study has concluded that zebrafish is a unique species which possesses desaturase gene with both Δ5 and Δ6 desaturase activities and elongase gene with high C_{18-20} elongase activities. In addition, Δ6 desaturase and putative desaturase genes of carp, salmon, cod, tilapia, seabream and rainbow trout species have been cloned (Seilez *et al.*, 2001, 2003; Zheng *et al.*, 2004a). As for the elongase gene, it has been cloned for Zebrafish, Atlantic salmon, Nile tilapia, African catfish and cod (Hastings *et al.*, 2001, 2004; Agaba *et al.*, 2005).

2.3 Lipid and fatty acids requirements in fish

The essential fatty acids requirements differ considerably between marine and freshwater fish species, and differs between inter and intra species in the same environment. The usage distribution of essential fatty acids also differs for various functions in the fish like normal growth and development including reproduction and its immune system.

Fatty acids act as precursors for a number of biologically active molecules like ecosanoids, pheromones, growth regulator and hormones (Pereira *et al.*, 2003). DHA and EPA are the major HUFA components of the cell membranes in fish whereas ARA is a minor component. Fish have correspondingly higher dietary requirements for *n*-3 HUFA as fish tissues have higher concentrations of DHA and EPA compared to ARA. Nonetheless, ARA should not be ignored because they play important roles in the formation of eicosanoids (Sargent *et al.*, 1999).

By and large, the role of HUFA is in maintaining the structural and functional integrity of cell membranes. Phosphoglycerides and their fatty acid compositions have a major and well-established role in maintaining the structure and function of cellular biomembranes. As oxygenated derivatives of HUFA produced from membrane phospholipids by the action of phospholipases, cyclooxygenases and lipoxygenases; eicosanoids have a range of highly active C_{20} compounds formed in trace amounts by virtually every tissue (Ganga *et al.*, 2005). Eicosanoids have a wide range of physiological actions, for example in blood clotting, immune response, inflammatory response, cardiovascular tone, renal function, neural function and also reproduction (Tocher, 2003). ARA is the major precursor of eicosanoids in fish involving cardiovascular functions, osmoregulation and functions of reproductive systems (Cejas *et al.*, 2004). EPA may also form eicosanoids but they are less biologically active than those formed from ARA. Besides, EPA competitively inhibits the formation of eicosanoids from ARA and as a result, the eicosanoids by EPA competitively interfere with the actions of eicosanoids formed from ARA (Sargent *et al.*, 1999). DHA especially is of importance in neural tissues as the dietary deficiency of DHA led to larval herring having impaired ability to capture prey at natural light intensities (Bell *et al.*, 1995) and impaired schooling behavior in yellowtail and Pacific threadfin (Masuda *et al.*, 1998; Ishizaki *et al.*, 2001). These studies entail that DHA has a critical role in the functioning of neural tissues in fish such as brain and eyes (Tocher, 2003).

2.3.1 Requirements in fish reproduction

Inadequate provision of essential fatty acids will cause low growth rate, poor food conversion rate and will affect reproductive performances of broodstock. As

it is, broodstock nutrition is one of the vital limiting factors for fish reproduction, egg and larval quality. Broodstock nutrition has significant effects on gonadal growth and fecundity (Mourente and Odriozola, 1990). Problems encountered in aquaculture such as reproductive deficiencies and production of low quality newly-hatched larvae are directly related to dietary regime and nutrients provided to broodstock (Izquierdo *et al.*, 2001). A study on the effect of dietary fatty acids on cultured and wild sweet smelt (*Plecoglossus altivelis*) broodstock gonads indicated the accumulation of DHA and EPA in ovary and testis despite being fed diets deficient in HUFA. This shows that the fish managed to synthesize the required fatty acid components and preferentially accumulated them in ovary and testis (Jeong *et al.*, 2002). Several studies also reported that dietary HUFA had significantly affect fecundity, fertilization rates, hatching and viability of eggs, embryonic development and larval growth (Fernandez-Palacios *et al.*, 1995; Izquierdo *et al.*, 2001).

Broodstock fish accumulate lipid reserves to be used either as energy source or for gametogenesis and gonadal maturation, but not every species uses similar organs for lipid deposition and also there are differences in the preference of the organ from which it will be mobilized. The female common torpedo (*Torpedo torpedo*) uses muscular and hepatic lipid for energy resources during vitellogenesis, the female bondella (*Coregonus macrophalmus*) uses perivisceral adipose tissue for gonadal development, while the female capelin (*Mallotus villosus*) catabolizes lipid from muscle during gonadal development. All fatty acids are not utilized in the same way during reproduction, for example the female capelin catabolized mainly monounsaturated fatty acids to provide energy whilst the HUFA are transferred to the eggs (Chatzifotis *et al.*, 2004). In the female gilthead seabream (*Sparus aurata*), a study to evaluate mobilization of lipids from muscle and liver towards the gonads in

aid of oocyte development had revealed evidence on depletion of liver and muscle lipid contents while gonad lipid content remained constant. This shows that the mobilization of lipids from other tissues contributed to the conservation of the gonad lipid content involving the mobilization of all fatty acid groups (Jerez *et al.*, 2006).

During maturation and reproduction, lipids are transported into oocytes from maternal reserves, stored and accumulated in yolk to meet energy and nutrient requirements before subsequently utilized by developing embryos (Brooks *et al.*, 1997). A study demonstrated that the percentage of lipid in ovaries of two live bearing fishes, Eastern mosquitofish (*Gambusia holbrooki*) and Sailfin molly (*Poecilia latipinna*) decreased during gestation period, indicating lipid was metabolized by embryos during development. It also showed that greater percentage of overall body lipids were moved to the ovaries when the fish clutches were large (Meffe and Snelson, 1993).

Although transportation of lipid has been studied, very little is known about the existence, role and regulation of the HUFA synthesis system throughout the follicle maturation process in the ovary (Wiegand, 1996). It is of considerable interest to seek detailed understanding on the actual role of HUFA; ARA, EPA and DHA in oocyte maturation, ovulation, spawning, hatchability and larval quality (Sorbera *et al.*, 2001; Bell and Sargent, 2003; Patino *et al.*, 2003)

2.4 Zebrafish (*Danio rerio*, Hamilton-Buchanan 1822)

2.4.1 General Information

The zebrafish are minnows, tropical freshwater fish widely distributed in India, Pakistan, Bangladesh, Nepal and Myanmar. It belongs to the family Cyprinidae and can be found in warm waters (24-35°C) with a pH range of 6.0-8.0.

Its habitats are slow-moving to stagnant body of water like streams, canals, ponds, irrigation channels and rice fields. Average size for adults range for 4 to 6 cm in size and it has five uniformly, pigmented, horizontal stripes on the side of the body, all extending onto the end of caudal fin rays; hence the name (FishBase, 2009). It is an omnivorous fish and its natural diet consists of insects, nematodes and algae (McClure *et al.*, 2006). This species is popular as aquarium fish and is common in aquarium shops worldwide.

2.4.2 Reproduction

Both male and female zebrafish are sexually mature at the age of 4 to 5 months old, is fusiform in shape but the adult male body is more streamlined and has yellow tinted ventral side and fins; whereas the adult female has a larger, whitish belly (Schilling, 2002). Breeding can be stimulated throughout the year and the fish is able to spawn in the interval of 2-5 days, but studies have shown that it can also spawn on a daily basis (Selman *et al.*, 1993; Lawrence, 2007). This species is asynchronous batch spawners, females scattering clutches of eggs over the substratum with hundreds of eggs in each clutch and no parental care. Upon release from the female, development of fertilized embryos will proceed and almost immediately become transparent; however without the presence of sperm growth will stop after the first few embryonic cleavages and then become opaque. This transparent fertilized egg is an important characteristic that makes zebrafish as a convenient research model. Development rapidly progresses, with precursors to all major organs appearing within 36 hours of fertilization. Hatching will take place anywhere from 48–72 hours post-fertilization, depending on the quality of the embryo itself and the environments (Kimmel *et al.*, 1995).

2.4.3 Gonad development and anatomy of the ovary

Gonad development pattern in zebrafish identifies this species as juvenile hermaphrodite. At the age of 4 weeks post-fertilization (pf), more than 80% of the juveniles possessed paired gonads with meiotic germ cells which represent presumptive ovaries. Starting week 5 pf, some fish displayed alterations of gonad morphology; which includes a decrease in number and size of oocytes, irregular oocyte shapes and an enhanced basophilia, and finally their degeneration into residual bodies. With the decline in oocyte number, stromal cells became more numerous and they infiltrated the gonadal matrix; which presumptive testes appear in week 7 pf. By week 11 pf, the sexual differentiation between males and females are prominent with the presence of well-developed ovaries and testes observed and the male to female ratio is approximately 1:1 (Maack, 1964; Maack and Segner, 2003). Table 2.1 is a more detailed summary of gonad morphology development and sexual differentiation in the zebrafish.

A well-developed ovary of a female zebrafish is bilobed. It is of the cystovarian type; paired primordia fused during the formation of the ovary and encloses a portion of the coelomic cavity. Eggs are ovulated into an ovarian lumen which is connected to a short oviduct that continues to a genital opening posterior to anus. The ovarian wall is smooth muscle covered by a thin epithelium, which overlaps a connective tissue compartment that projects as longitudinal folds or ovigerous lamellae, into the ovarian cavity. This ovigerous lamellae contains randomly arranged follicles in different stages of development as well as atretic follicles and postovulatory follicles. Oogonia and the earliest meiotic oocytes are embedded directly in ovarian stroma. Growing oocytes resides within ovarian follicles where each ovarian follicles is a make up of an oocyte enveloped by layers of somatic cells (Selman *et al.*, 1993).

Table 2.1: Summary of gonad morphology development and sexual differentiation in the zebrafish (Maack, 1964; Maack and Segner, 2003)

Age(week)/ Total length of larvae (mm)	Gonad Observation
2	• Gonad is undifferentiated in coelomic cavity in caudo-dorsal position. • It contains 10-20 perimordial germ cells, surrounded by small rim of somatic cell. • The perimordial germ cells is ±12μm in diameter with-nucleus 6-10μm in diameter with prominent nucleolus.
4	• Gonad is extended in anterior-posterior position. It contains germ cells with large nuclei and small cytoplasmic rim. • There are 2 types of germ cells can be discerned; type 1: oogonia, and type2: oocytes at chromatin-nucleolar stage
5 (10-15mm)	• Female ovaries are now a mix of germ cells type 1, type 2 and perinucleolar stage oocytes. There is an-increase in oocyte sizes because of ooplasm enlargement. • Follicular cells start to develop around oocytes.
6 (9-17mm)	• Ovaries are larger and contain mostly well differentiated perinucleolar oocytes. Undifferentiated germ cells type 1and type 2 can only be found at cranial and caudial ends. • There are altered ovaries detected in some individuals. In altered ovaries, oocyte and nucleus size are reduced while the cell and nucleus shape become increasingly irregular.
7 (13-22mm)	• This is the age where sex differentiation begins. • Ovaries morphological appearances: (i)large and well developed (ii)small and altered histological features. • In altered ovaries; reduced frequency of fibroblast-like and macrophagic non-germ cells and elevated number of undifferentiated germ cells (correspond to germ cells type1) It resemble spermatogonial cysts, and often presence of lumen in center of germ cells group is detected.
8 (13-23mm)	• There is more pronounced variation of gonad morphology and also reduced percentage of fish with well-differentiated ovaries, with more fish showing gonads with altered ovaries and early testes.
9-11 (12-27mm)	• By week 10, fish shows either well-differentiated ovary or testis. • Ovaries were further developed with oocyte size enlarged due to increasing cytoplasm volume.
11-12	• Mature gonads.

2.4.4 Stages of oocyte development

The development of zebrafish oocytes were grouped into five stages based on prominent morphological characters and sizes. Selman *et al.* (1993) did a comprehensive study on the five stages of zebrafish oocyte development or follicle maturation. In a nutshell, the five stages are (i) stage I: primary growth, (ii) stage II: cortical alveolus stage, (iii) stage III: vitellogenesis, (iv) stage IV: maturation, and (v) stage V: mature eggs or ovulation stage. A rough sketch on what consists of a follicle is shown in figure 2.2. Table 2.2 provides a summary of morphological changes during follicle maturation in the five oocyte development stages. This study has become a basic classification guideline for future zebrafish oocyte studies (e.g: Wang and Ge, 2004; Goto-Kazeto *et al.*, 2004; DiMuccio *et al.*,2005; Ings and Van Der Kraak, 2006), which authors have tailored to the needs of their research.

2.4.5 Zebrafish as a vertebrate model system

Zebrafish (*Danio rerio*) is currently the most popular fish model organism in developmental biology laboratories. It has several contributing factors as a model system which makes this species favorable for oogenesis study;

1) Zebrafish has small body size and is practically easy to maintain and prepare for large-scale breeding under suitable conditions (Brand *et al.*, 2002)
2) The female zebrafish has high fecundity and a productive spawner; with the oocytes easily differentiated to 4-5 distinct follicle stages throughout the follicle maturation process (Selman *et al.*, 1993; Gerhard and Cheng, 2002).

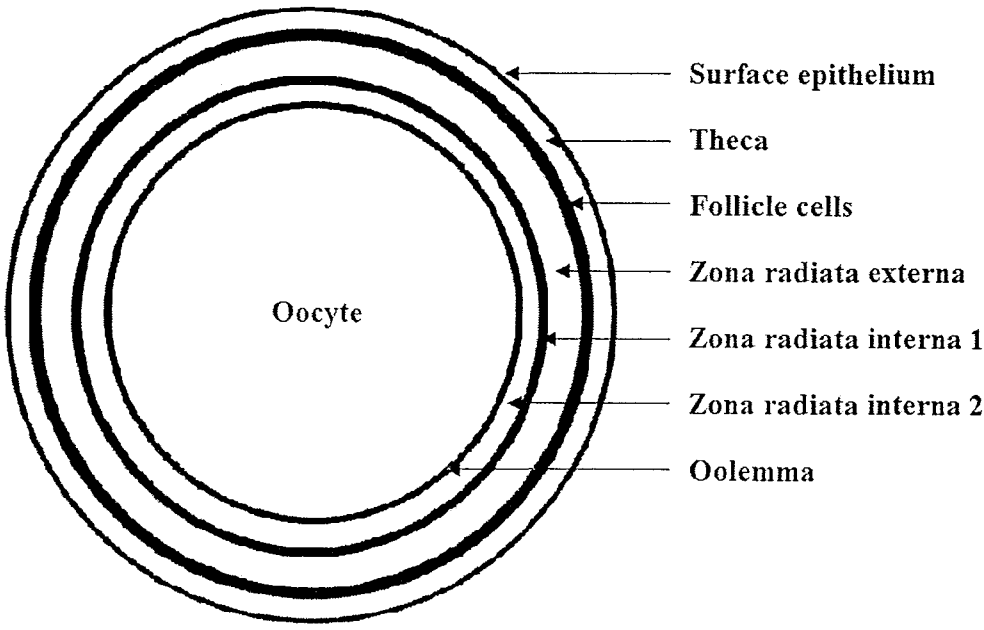

Figure 2.2: Oocyte and its surrounding follicle layers (adapted from Selman *et al.*, 1993).

Table 2.2: Summary of morphological changes during follicle maturation in the five oocyte development stages (Selman *et al.*, 1993).

Developmental stage	Morphological Observation
Stage I: (Primary growth)	Stage IA (pre-follicle phase): oocyte diameter= 7-20 μm • Small oocytes lie in nests that are isolated from the ovarian stroma and ovarian follicles by a single layer of pre-follicle cells. • Chromosomes are initially not visible within nuclei of early meiotic oocytes but gradually become noticeable during prophase. • During leptotene, the chromosomes undergo a chromatin-nucleolus phase. As the oocytes grow, the chromosomes continue to condense and become more visible. Before they reach diplotene, they are completely enveloped by a sheath of pre-follicle cells which leads to the separation of individual oocytes from the nest. Stage IB (follicle phase): follicle diameter= 20-140 μm • Fresh follicles are transparent with visible centrally located nucleus. • The oocyte is surrounded by a single layer of squamous follicle cells and the enlarging germinal vesicle contains many peripherally located nucleoli. Nucleoli display both internal fibrillar and external granular components. • Intracellular organelles proliferate and ooplasm becomes more basophilic. Shortly after entering stage IB, oocyte enters diplotene and arrest. • After leaving the nest,the oocyte is enveloped by layers of somatic tissues and the definitive follicle is formed. This consists of an oocyte surrounded by a single layer of squamous follicle cells, which is surrounded by the theca, a vascularized connective tissue compartment. • The theca in turn is covered by a surface epithelium. The oocyte surface has short microvilli that extend toward the overlying follicle cells which also has microvilli extending towards the oocyte. This is the earliest manifestation of the vitelline envelope or zona radiata.
Stage II: (Cortical Alveolus)	Follicle diameter= 0.14-0.34 mm • Oocytes become opaque and centrally located germinal vesicles are difficult to distinguish. • The emergence and proliferation of cortical alveoli (yolk vesicles) within oocyte. Cortical alveoli are membrane-limited vesicles of variable size that stain with dyes for protein and carbohydrate. They appear in close proximity of Golgi complexes that participate in the synthesis of their contents. As oocyte grows, cortical alveoli increase in number and size, thus occupy much of the ooplasm. • During this stage, germinal vesicle continues to enlarge and becomes highly irregular in shape. Nucleoli proliferate, become most numerous by now and within ooplasm mitochondria elongate. By the end of this stage, lysosome-like bodies have become quite noticeable. • A prominent change that also occurs during stage II is the formation of tripartite vitelline envelope at the zona radiate. This newly formed vitelline envelope is perforated with pore canals which contains macrovilli from both oocyte and follicle cells.

Table 2.2: Continued.

Developmental stage	Morphological Observation
Stage II: (Cortical Alveolus)	• The three layers are referred as:- (i) zona radiata externa: homogenous outer layer near the follicle cells, (ii) zona radiata interna 1: newly formed inner layer and more electron lucent than the outer layer, (iii) zona radiata interna 2: thickest and most complex layer containing numerous horizontal laminae closest to the oolemma. • Overlaying the vitelline envelope, follicle cells continue to divide around the growing oocyte and become cuboidal. • Special theca cells which are thought to be steroid secreting cells appear.
Stage III: (Vitellogenesis)	Follicle diameter= 0.34-0.69 mm • Follicles become increasing opaque and the germinal vesicle is completely obscured. • Oocytes size increases due to protein accrual or accumulation of yolk, which involves the sequestration of vitellogenin via endocytosis and subsequently processed into yolk proteins that accumulate within membrane limited yolk bodies. Vitellogenin is a hepatically derived, female-specific, yolk-percursor protein. • Other changes that coincide with vitellogenesis are the vitelline envelope become thinner, the number of special thecal cells increases and become larger. • Both rough endoplasmic reticulum and lysosomes proliferate, while within the oocyte the germinal vesicle crenated outline becomes smoother and moves away from the central ooplasm. • Small nucleoli are noticeable in the centre and cortical alveoli gradually displaced toward the periphery as the yolk bodies accumulate at the central oocyte. • Towards the end of vitellogenesis, follicles are competent to respond to endogenous hormone thus ready for maturation.
Stage IV: (Maturation)	Follicle diameter= 0.69-0.73 mm • Germinal vesicles break down (GVBD) is a process when meiosis is reinitiated, the germinal vesicle migrates towards the oocyte periphery, the nuclear envelope breaks down, the first meiotic division occurs, and the chromosomes proceed to second meiotic metaphase where they stop. • The most notable difference when GVBD occurred is the opaque oocytes become translucent. Yolk bodies lose their crystalline main bodies and develop a homogenous interior. • Due to hydration, oocytes become enlarged.and the follicle cells retract from the oocyte prior to ovulation.
Stage V: (Mature egg/ Ovulation)	Follicle diameter= 0.73-0.75 mm • Translucent eggs are ovulated into ovarian lumen. No lipid droplets can be seen within eggs. • The eggs size is slightly smaller as the follicular wall surrounding unovulated eggs is approximately 10 μm.

An *in vitro* maturation method by inducing zebrafish oocytes with gonadotropin and steroid has been well established (Selman *et al.*, 1994; Pang and Ge, 2002b). This helps in monitoring oogenesis and folliculogenesis in real time, thus making the zebrafish a useful model to understand oogenesis and folliculogenesis (Ge, 2005).

Besides developmental biology, the usefulness of zebrafish have also been recognized in other fields which includes comparative phylogenetics (Metscher and Ahlberg, 1999), gerontology (Gerhard and Cheng, 2002), toxicology (Spitsbergen and Kent, 2003), and aquaculture (Dahm and Geisler, 2006). The fact that zebrafish is the only teleost to have a genome sequencing project that is currently underway shows the popularity of this species as one of the model organism in genomic studies (The *Danio rerio* Sequencing Project, 2009).

2.4.6 Gene expression studies on zebrafish oogenesis

Various molecular studies have been done to investigate the functions and roles of genes that affect fish oogenesis. Emphasis has been specially given on studying genes that promote oocyte maturation. Selman *et al.*, (1994) had notably facilitated the studying of oocyte maturation in zebrafish by establishing a reliable *in vitro* bioassay. The relative effectiveness of several steroids and their metabolites in eliciting oocyte maturation *in vitro* was studied and the steroid hormone 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) appeared to be the most effective in inducing oocyte maturation.

Oocyte maturation is the event in which fully grown oocytes undergoes germinal vesicle breakdown where it becomes translucent. Studies have shown that it is triggered by a maturation promoting factor (MPF) that consists of *cdc2* (a catalytic

subunit) and cyclin B (a regulatory subunit) and its binding activated phosphorylation on threonine 161 by *cdk7* (an activator of *cdc2*), in which cyclin B mRNA stored in immature zebrafish oocytes is translationally activated upon the stimulation of DHP, an event prerequisite for initiating oocyte maturation in this species (Kondo *et al.*, 1997, 2001).

Activin βA subunit and activin type IIA (ActRIIA) receptors are expressed in the zebrafish ovary, suggesting paracrine roles for activin in the ovarian functions. Activin significantly stimulated zebrafish oocyte maturation *in vitro*, and this effect could be blocked by follistatin, an activin-binding protein. Interestingly, follistatin also blocked the stimulatory effect of human chorionic gonadotropin (hCG) on the oocyte maturation. Results suggest that gonadotropin activates the activin system in the zebrafish ovary by increasing the expression of both activin and its receptors (Pang and Ge, 2002a). Further study shows that activin βA and βB exhibit distinct expression patterns during the development of the ovary and the daily ovarian cycle of the zebrafish. Activin βA seems to be involved in promoting ovary and follicle growth, whereas activin βB may have a tonic role throughout follicle development but becomes critical at the late stage of oocyte maturation and/or ovulation (Wang and Ge, 2004).

The growth differentiation factor 9 homolog (*gdf9*) is a gene with a unique oocyte-specific expression. Although this study shows that *gdf9* expressions gradually decreased during follicular development, conversely as an oocyte-derived growth factor, it is highly conserved across vertebrates (Liu and Ge, 2007). Another study shows increasing expressions for steroidogenic acute regulatory protein (StAR), P450 aromatase (P450aromA) and 17β-hydroxysteroid dehydrogenasetype 3 (17β-HSD3) during *in vitro* incubation of vitellogenic follicles in response to hCG.

This result suggests that gonadotropins play a key role in the regulation of StAR, P450aromA and 17β-HSD3 in zebrafish (Ings and Van Der Kraak, 2006).

Other genes such as *αE-catenin*, *β-catenin* and *plakoglobin* which form heterotypic adherens junctions between oocytes and follicle cells (Cerda. *et al.*, 1999); cytochrome P450 aromatase genes (*CYP19A1* and *CYP19A2*) which are responsible for the conversion of androgens to estrogens (Goto-Kazeto *et al.*, 2004); and membrane-bound progestin receptors genes (*mPRα*, *mPRβ* and *mPRγ*) which are potential intermediaries in meiotic maturation of fish oocytes (Kazeto *et al.*, 2005), are all vital in the zebrafish reproduction system. However, none of the genes described above display direct association with HUFA biosynthesis in zebrafish oocytes.

2.5 Normalization of gene expression

The semi-quantitative real time RT-PCR analysis chosen for this study quantifies PCR product during the log phase of the reaction. This method requires equal input amounts of RNA for all samples. In order to obtain standardized quantitative results, an endogenous housekeeping gene, which is present at constant amounts in all samples, can be used to correct for variations in input RNA amounts and inefficiencies in cDNA synthesis; and results were normalized to these values (Overbergh *et al.*, 1999). The accepted normalization method that is used widely in genomic studies is gene of interest expression value over housekeeping gene expression value ratio.

Commonly used housekeeping genes such as *beta-actin*, *18S ribosomal RNA* (*18S rRNA*), *acidic ribosomal phosphoprotein* (*ARP*), *elongation factor-1 alpha* (*EF1-α*) and *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) have been